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Complete List of Authors:	Young, Vicky Jane ; University of Edinburgh, MRC Centre for Reproductive Health Ahmad, Furquan; University of Edinburgh, MRC Centre for Reproductive Health Duncan, Colin; University of Edinburgh, MRC Centre for Reproductive Health Horne, Andrew; University of Edinburgh, MRC Centre for Reproductive Health
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The role of TGF-β in the pathophysiology of peritoneal endometriosis

Authors

Vicky J Young, S F Ahmad, W Colin Duncan, Andrew W Horne

Address

MRC Centre for Reproductive Health, The University of Edinburgh, Queen's Medical Research Institute, Edinburgh EH16 4TJ, UK

Corresponding author

Professor Andrew W Horne

andrew.horne@ed.ac.uk

Tel: +44 (0)131 242 6796

Fax: +44 (0)131 242 6441

MRC Centre for Reproductive Health, Queens Medical Research Institute, The University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

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Abstract

BACKGROUND: Endometriosis is estimated to affect 6-10% of women of reproductive age and it is associated with chronic pelvic pain, dysmenorrhea and subfertility. It is currently managed surgically or medically but symptoms recur in up to 75% of cases and available medical treatments have undesirable side effects. Endometriosis is defined as the presence of endometrial tissue outside the uterus with lesions typically found on the peritoneum. The aetiology of endometriosis is uncertain but there is increasing evidence that transforming growth factor (TGF)-β plays a major role.

OBJECTIVE AND RATIONALE: A descriptive review was undertaken of the published literature on the expression pattern of TGF-β ligands and signalling molecules in women with and without endometriosis, and on the potential roles of TGF-β signalling in the development and progression of peritoneal endometriosis. The current understanding of the TGF-beta signaling pathway is summarized.

SEARCH METHODS: We searched the Pubmed database using the terms 'transforming growth factor beta' and 'endometriosis' for studies published between 1995 and 2016. The initial search identified 99 studies and these were used as the basic material for this review. We also extended our remit for important older publications. In addition, we searched the reference lists of studies used in this review for additional studies we judged as relevant. Studies which were included in the review focused on peritoneal endometriosis only as increasing evidence suggests that ovarian and deep endometriosis may have a differing pathophysiology. Thus, a final 95 studies were included in the review.

OUTCOMES: TGF-β1 is reported to be increased in the peritoneal fluid, serum, ectopic endometrium and peritoneum of women with endometriosis compared to women without endometriosis, and TGF-β1-null mice have reduced endometriosis lesion growth when compared to their wild-type controls. Studies in mice and women have indicated that increasing levels of TGF-β ligands are associated with decreased immune cell activity within the peritoneum, together with an increase in ectopic endometrial cell survival, attachment,

invasion and proliferation, during endometriosis lesion development. TGF-β1 has been associated with changes in ectopic endometrial and peritoneal cell metabolism and the initiation of neoangiogenesis, further fuelling endometriosis lesion development.

WIDER IMPLICATIONS: Together these studies suggest that TGF-β1 plays a major role in the development of peritoneal endometriosis lesions and that targeting this pathway may be of therapeutic potential.

Keywords
endometriosis, endometrium, peritoneum, smad, immune cells, angiogenesis

INTRODUCTION

Endometriosis is estimated to affect 6-10% of women of reproductive age and is associated with chronic pelvic pain, dysmenorrhea, dyspareunia and subfertility (Meuleman et al. 2009; Giudice & Kao 2004). These symptoms affect general physical, mental and social well-being and have a significant impact on quality of life (Dunselman et al. 2014). Endometriosis is currently diagnosed by laparoscopy, but the time to diagnosis can be long (on average 6-7 years) owing to the variability of the symptoms and a lack of diagnostic biomarkers (Nnoaham et al. 2011). Symptoms can be managed medically or surgically but symptoms reoccur in up to 75% of surgical cases within 2 years and available medical treatments have undesirable side effects and are contraceptive (Jacobson et al. 2009). The annual average health care cost associated with endometriosis in the UK is estimated at £8.5 billion, which is similar to that of diabetes and rheumatoid arthritis (Simoens et al. 2012).

Endometriosis is a benign, estrogen-dependent disorder defined as the presence of endometrial glands and stroma outside the uterine cavity (Giudice 2010). It is now generally accepted that there are three distinct types of endometriosis: peritoneal, ovarian and deep endometriosis, each of which is thought to have a different pathogenesis (Nisolle & Donnez 1997). The most common type of endometriosis is peritoneal endometriosis and this is the focus of our review (Mahmood & Templeton 1991).

The widely accepted hypothesis for the development of endometriosis is the retrograde menstruation theory proposed by Sampson in 1927. This theory suggests that during menstruation viable endometrial tissue is refluxed through the Fallopian tubes into the peritoneal cavity where it implants and grows (Sampson 1927). Sampson's theory is supported by the high prevalence of pelvic endometriosis in girls with congenital menstrual outflow obstruction and the distribution of lesions in the abdominal cavity (Nap 2004). It is also supported by the fact that women with endometriosis have more frequent sub-endometrial myometrial contractile waves than women without endometriosis (Salamanca &

Beltrán 1995) In addition women with endometriosis have higher volumes of refluxed menstrual blood than healthy controls (Halme et al. 1984; Salamanca & Beltrán 1995). However, as retrograde menstruation is seen in over 90% of women, this hypothesis fails to fully explain why shed endometrial tissue implants in some women and not in others (Halme et al. 1984).

It is now agreed that a combination of genetic, hormonal, immunological and anatomical factors contribute to the formation and development of endometrial lesions (Giudice & Kao 2004). The formation of peritoneal lesions has been attributed to the attachment of ectopic endometrium to the peritoneal surface, invasion of the peritoneum, neoangiogenesis, suppression of the immune system and continued survival and growth of lesion tissue (Giudice & Kao 2004; Young et al, 2013). Increased concentrations of inflammatory cytokines and growth factors within the peritoneal fluid and peritoneal tissue are thought to contribute to peritoneal lesion formation (Young et al. 2013). Transforming growth factor beta (TGF-β) is an inflammatory growth factor that regulates a variety of cellular functions including cell adhesion, invasion and angiogenesis, all of which are essential during endometriosis lesion development. Levels of TGF-β are reported to be increased in the peritoneal fluid, serum, ectopic endometrium and peritoneal tissue of women with endometriosis compared to controls (Oosterlynck et al. 1994; Pizzo et al. 2002; Chegini et al. 1994; Young et al. 2014a; Young et al. 2014b) and Tgfb1 null mice have reduced endometriosis lesion growth when compared to wild-type controls (Hull et al. 2012), suggesting TGF-β1 plays a key role in lesion development. Nevertheless, the functional role that TGF-β plays in the pathophysiology of endometriosis is less clear. This review will attempt to highlight the expression pattern and potential roles of TGF-β ligands and signalling in the pathophysiology of peritoneal endometriosis.

METHODS

We searched the Pubmed database using the terms 'transforming growth factor beta' and 'endometriosis' for studies published between 1995 and 2016. The initial search identified 99 studies and these were used as the basic material for this review. We also extended our remit for important older publications. In addition, we searched the reference lists of studies used in this review for additional studies we judged as relevant. Studies which were included in the review focused on peritoneal endometriosis only as increasing evidence suggests that ovarian and deep endometriosis may have a differing pathophysiology. Thus, a final 95 studies were included in the review.

RESULTS

The TGF-β signalling pathway

The TGF-β superfamily consists of over 30 different ligands in humans and includes three TGF-β isoforms, four activin isoforms, 10 bone morphogenetic protein isoforms, 11 growth and differentiation factor isoforms and the protein nodal (Schmierer & Hill 2007). TGF-β is secreted in a latent complex consisting of three proteins: TGF-β, an inhibitor (latency-associated protein, LAP, which is derived from the TGF-β propeptide) and an extracellular matrix (ECM)-binding protein (latent TGF-β binding proteins, or LTBPs). LTBPs interact with fibrillins and other ECM components and thus function to localize latent TGF-β in the ECM. LAP contains an integrin-binding site (RGD), and several RGD-binding integrins are able to activate latent TGF-β through binding this site (Munger and Sheppard 2011). A common pathway for TGF-β activation is through integrins; αV-β6 on the surface of epithelial and mesothelial cells induces a conformational change by binding to the RGD motif present in LAP and activate TGF-β, inducing adhesion-mediated cell forces that are translated into biochemical signals which can lead to liberation/activation of TGF-β from its latent complex (Munger et. Al. 1999, Munger and Sheppard 2011). Secondly, αV-β6 integrin on the

surface of epithelial and mesothelial cells can activate latent TGF-β by creating a close connection between the latent TGF-β complex and matrix metalloproteinase (MMP)-2 and MMP-9, which can activate TGF-β through proteolytic degradation of the LAP (Yu & Stamenkovic 2000; Mu et al. 2002; Annes 2003; Wipff and Hinz 2008). Notably integrin αV and β6 null mice both display similar phenotypes to the Tgfb1 null mice (Bader et al. 1998; Huang et al. 1996; Shull et al. 1992). In addition to MMPs, other proteases, including plasmin, have been shown to activate TGF-β ligands through proteolytic degradation (Yu & Stamenkovic 2000; Annes 2003), together with other factors including an acidic pH, which denatures the LAP (Lyons et al. 1988), and thrombospondin-1, which induces a conformational change in LAP thus leading to activation of TGF-β ligands (Schultz-Cherry & Murphy-Ullrich 1993), Additional pathways may also lead to the activation of TGF-β ligands, and the diverse range of TGF-β activation pathways demonstrates that this is a key step in the regulation of TGF-β signalling. Annes has published a comprehensive review on TGF-β activation and regulation, which describes these processes and their importance in more depth (Annes 2003).

Classically, activated TGF-β ligands bind to the constitutively active transmembrane receptor, TGF-β receptor II (TGF-βRII), which induces a conformational change and initiates the recruitment of transmembrane TGF-β receptor I (TGF-βRI) (Figure 1) (Shi & Massague 2003). The TGF-β receptor complex then in turn phosphorylates the receptor regulated transcription factors *SMAD2* and *SMAD3* (Figure 1) (Shi & Massague 2003). A third TGF-β receptor, TGF-β receptor III (TGF-βRIII), has been described and was originally thought to be a TGF-β co-receptor, presenting TGF-β ligands to TGF-βRII (Cheifetz et al. 1988). More recently, it has been shown that Tgfb-RIII null mice die at gestational day 13.5 indicating TGF-βRIII to be an essential component of the TGF-β signalling pathway in development (Compton et al. 2007). However, little is known about the role of this receptor in TGF-β signalling.

Phosphorylated receptor Smads form a heteromeric complex of two receptor Smads together with the co-Smad, Smad4, before nuclear translocation and regulation of transcriptional responses (Figure 1) (Schmierer & Hill 2007). Smad-mediated transcription can be either positive or negative and is thought to occur through chromatin remodelling and histone modification rather than direct recruitment of transcriptional machinery (Ross et al. 2006; Shi & Massague 2003). Inhibitory Smad7 mediates negative feedback in the TGF-β signalling pathway by competing for TGF-β receptor I binding and inhibiting phosphorylation of Smad2 or Smad3 (Schmierer & Hill 2007). TGF-β signalling through Smad independent pathways, such as tyrosine kinase and G-protein-coupled signalling pathways, has been described, although the links between the activated TGF-β receptors and the downstream signalling molecules remain unknown in most cases (Moustakas 2005). Additionally, TGF-β signalling through the nodal signalling pathway, a crucial embryogenesis pathway, has been described in tumorogenesis (Quail et al. 2013; Schmierer & Hill 2007; (Moustakas 2005).

TGF-β signalling elicits a wide variety of downstream processes, however this is in direct contrast with the number of Smad proteins recruited by the TGF-β receptors and it is not fully understood how TGF-β ligands can produce a variety of distinct responses (Shi & Massague 2003). Several theories exist that attempt to explain these responses. Firstly; it has been reported that distinct signal intensities can stimulate differential gene expression, for example, the nuclear concentration of a transcriptional activator required for expression is determined by the binding affinity of a target gene promoter (Schmierer & Hill 2007). Secondly, differing concentrations of TGF-β ligands can activate different responses in gene expression (Schmierer & Hill 2007). Thirdly, the establishment of reciprocal gradients of repressor gene expression have been reported for some genes. Schmierer and Hill describe these processes in more detail (Schmierer and Hill 2007). More recently, a cell-type-specific master transcription factor which directs different responses to Smad2 or Smad3 in different cell types has been reported (Mullen et al. 2011). The mechanism that determines phosphorylation

of Smad2 over Smad3, or vice-versa, by the TGF-β receptor in a particular cell type is not yet known (Shi & Massague 2003).

TGF-β expression in women with peritoneal endometriosis

Several studies have reported significantly higher levels of TGF-β1 in serum, peritoneal fluid, peritoneum and eutopic endometrial tissue of women with endometriosis when compared to women without endometriosis, suggesting that altered TGF-β expression and/or signalling may contribute to the pathophysiology of endometriosis (Oosterlynck et al. 1994; Chegini et al. 1994;Kupker et al. 1998; Pizzo et al. 2002; Fan et al. 2005; Young et al. 2014a; Young et al. 2014b).

Peritoneal mesothelial cells are the largest cell population within the peritoneal cavity and are reported to overexpress TGF-β, and in particular TGF-β1 ligands, into the peritoneal fluid in response to peritoneal related pathologies, such as fibrosis and peritoneal cancers, suggesting that they may play a significant role in the elevated levels of TGF-β1 found in women with endometriosis (Offner et al. 1996). Recently, we have described the peritoneal mesothelial cells as a source of TGF-β1 in the pathology of endometriosis through a series of immunohistohemical staining on primary human peritoneal biopsies and through studies *in vitro* of primary peritoneal mesothelial cells (Young et al. 2014b). Additional sources of peritoneal fluid TGF-β in women with endometriosis are thought to be from shed menstrual tissue, ectopic endometrial cells and macrophages (Omwandho et al. 2010). The peritoneum from women with endometriosis has been reported to express significantly higher levels of TGF-β1, TGF-β3 and Smad3 than the peritoneum from control women with benign ovarian tumours (Li et al. 2011). However the nature of the cells contributing to this increase (either immune cells, nerve cells, endothelial cells or mesothelial cells), is not clear (Li et al. 2011). Furthermore, as the control group of women included in this study presented with benign ovarian tumours, it is not clear if the observed differences in TGF-β ligand and Smad3

expression were linked to the presence of endometriosis or the presence of ovarian pathology (Li et al. 2011). We have recently described a significant increase in TGF-β1 mRNA expression in the peritoneum adjacent to endometriosis lesions, when compared to peritoneum from sites distal to lesions in women with endometriosis. We found no change in mRNA expression of TGF-β signalling components (TGF-β receptors 1, 2 and Smad3) in the same tissue set, suggesting that the local increase in TGF-β1 may have downstream consequences on TGF-β signalling targets within the peritoneum (Young et al. 2014b).

TGF-β1, 2 and 3 are expressed in the human endometrium and their expression is cyclically regulated, with all 3 isoforms being expressed during menstration and found in shed endometrial tissue. Immunohistochemical analysis showed that TGF-β1 was localised within the stromal cells, glandular cells and macrophages of endometrial tissue, and TGF-β2 and 3 have been localised to the stromal cells and glandular cells of the endometrium (Chegini et al. 1994; Johnson et al. 2005).. Additonally, TGF-β1 protein levels are significantly increased in the nerve fibres of peritoneal endometriosis lesions, when compared to nerve fibres in peritoneum from women without endometriosis, and a statistically significant relationship was found between TGF-β1 expression and dysmenorrhea (Tamburro et al. 2003).

Despite conclusive evidence that TGF-beta isoforms are expressed and play a crucial signalling role in human endometrium, there is no literature directly showing TGF-beta expression and localisation to endometriosis lesion tissue. It is also not yet known if the increased levels of TGF-β1 in the peritoneal fluid of women with endometriosis precedes or follows the development of endometriosis. However, as retrograde menstruation and the presence of endometrial cells within the peritoneal cavity can induce inflammation and TGF-β is an inflammatory cytokine, the development of endometriosis and the increase in TGF-β1 are likely to go hand-in-hand (D'Hooghe et al. 2001a; D'Hooghe et al. 2001b; Li et al. 2011).

Only two of the reported studies indicated whether total or bioactive levels of TGF-β1 were measured, with both reporting only total levels to be measurable in peritoneal fluid, suggesting TGF-β ligands are activated locally, and therefore it is important to investigate the local changes induced by the presence of endoemtriosis lesions in the activation of TGF-β (Oosterlynck et al. 1994; Young et al. 2014a). One study has examined activation of TGF-β in women with endometriosis, and this was via the plasminogen activation pathway, which the authors found to be increased at sites of endometriosis lesions, suggesting that there may be more TGF-β activity in endometriosis lesions and the surrounding peritoneum (Komiyama et al. 2007). Several other activation pathways are likely to play a role in peritoneal TGF-β ligand activation and may be altered in women with endometriosis. Peritoneal mesothelial cells and endometriosis lesions express several integrins, including integrin αV and β6 which are known activators of TGF-β ligands, as described above (Odor 1954; Bardi & Hope 1964; van der Linden et al. 1994). These factors may contribute to the activation of TGF-β ligands within the local peritoneal enviroment and changes in integrin expression in women with endometriosis may lead to an increase in TGF-β activity. However, despite this pathway being a credible mechanism for TGF-β ligand activation in women with endometriosis, it has not yet been investigated in the pathophysiology of endometriosis.

TGF-β1 levels may be cyclically regulated within the peritoneal fluid of women and levels of TGF-β1 are significantly increased in the peritoneal fluid of women with endometriosis when compared to women without disease (Kupker et al. 1998; Pizzo et al. 2002; Oosterlynck et al. 1994; Young et al. 2014a; Young et al. 2014b). Recently, we reported TGF-β2 and TGF-β3 to be present within the peritoneal fluid, however levels of these ligands remained unchanged between women with and without endometriosis (Young et al. 2014b).

Interestingly, only two studies have quantified serum levels of TGF-β in women with endometriosis compared to women without. Pizzo et al. (2002) examined the levels of TGF-β

using ELISA in serum and peritoneal fluid isolated from 26 women with endometriosis and described a significant increase in serum-TGF-β concentrations, which increased with the severity of the disease and in a similar fashion to peritoneal fluid levels of TGF-β. However, this study made no distinction between TGF-β isoforms measured and it is not clear if this is TGF-β1 or all TGF-β ligands (Pizzo et al. 2002). Another study from a different group where authors have investigated the association between endometriosis and TGF-β1 gene polymorphisms using restriction fragment length polymorphism analysis and serum TGF-β1 levels in Korean women, independentaly confirmed that serum TGF-β1 levels were significantly higher in Korean women with endometriosis (n=120) than in controls (n= 89) (Lee et al. 2011). Both studies described a significant increase in TGF-β or TGF-β1 in the serum of women with endometriosis compared to controls, suggesting TGF-β may be a potential biomarker for the detection of endometriosis.

Endometriotic lesions express TGF-β1, 2 and 3, in differing protein concentrations, with TGF-β1 being the most abundantly expressed TGFβ protein isoform (Chegini et al. 1994). TGF-β1 was shown to be expressed in all cell types, except endometrial stromal cells, found within surgically induced endometriosis lesions in a rat (Chegini et al. 1994). One study demonstrated TGF-β mRNA expression to be increased in endometriosis lesion tissue when compared to eutopic endometrial tissue, however it is not clear if the endometrial control tissue is from women with or without endometriosis and the TGF-β isoforms measured are not reported (Fan et al. 2005). The TGF-β signal transducers Smad3, pSmad3, and Smad4, and the inhibitory Smad7 proteins were also observed in the endometrial stromal and epithelial cells (Luo et al., 2003a) and suggest a role for TGF-βs in the normal function of the human endometrium. In eutopic endometrium transcriptional activity of Smad3 is suppressed by the estrogen receptor (ER) in an estradiol-dependent manner, and ER-mediated transcription increases after activation of TGF-β signaling (Matsuda *et al*., 2001; Cherlet and Murphy, 2007). Studies have also shown that eutopic endometrium express Smads and that

TGF-β1 increases both the expression of Smad3, and the phosphorylation of Smad3 *in vitro* in a dose-dependent manner, suggesting endometriotic cells may also be responsive to TGF-β1 signalling (Luo et al. 2003b). TGF-β1 was shown to be aberrantly expressed in the endometrium of women with endometriosis when compared to women without endometriosis, an observation the authors suggested may be linked to the increased cell proliferation seen in the endometrial cells of women with endometriosis (Johnson et al. 2005).

Recently, Hull et al. described a reduced growth of endometriosis lesions in TGF-β1-null mice when compared to their wild-type counterparts, demonstrating TGF-β1 to play a key role in endometriosis lesion development (Hull et al. 2012) and tissue repair and remodelling (Hull et al. 2008). These studies have been summarised in Table 1.

A role for TGF-β1 in the pathophysiology of peritoneal endometriosis

Although TGF-β1 expression appears to be increased in women with endometriosiscompared to women without endometriosis, less is known about the functional role of TGF-β1 in the development and maintenance of peritoneal endometriosis. TGF-β1 is a multifunctional cytokine, which is known to regulate a variety of biological processes e.g. cell proliferation, ECM formation, tissue remodeling, and inflammation (Massague et al. 2000; Jakowlew 2006). Similar biological events occur during endometriotic lesion establishment, and although there is little understanding of the signaling events that control them, there is evidence of TGF-β1 involvement. Herein follows a review of the possible functional roles for TGF-β1 in the pathophysiology of peritoneal endometriosis. These functions are summarised in Table 2.

TGF-β regulation of ectopic endometrial cell survival

TGF-β1, together with its downstream signalling targets involved in cell survival, including mRNA expression levels of *BAX* and *C-MYC*, were shown to be altered in the eutopic

endometrium of women with endometriosis compared to women without disease (Johnson et al. 2005), suggesting that increased TGF-β1 may lead to increased apoptosis resistance in the shed endometrial tissue of women with endometriosis. The change in *BAX* and *C-MYC* expression may facilitate survival of ectopic endometrial tissue during transport to the peritoneal cavity (Johnson et al. 2005). Furthermore, the increasing concentrations of TGF-β1 in the peritoneal fluid of women with endometriosis may further contribute to the expression of anti-apoptotic factors in shed endometrial tissue (Seoane 2006). Tgfb1 null mice showed reduced numbers of endometrial epithelial cells, without any observed changes in cell proliferation, leading to the hypothesis that TGF-β1 may be responsible for inducing anti-apoptosis effects within these cells, supporting this theory (Hull et al. 2012).

Together with increased apoptosis resistance and decreases in immune cell numbers and activity within the peritoneal fluid and peritoneum, TGF-β may also contribute to ectopic tissue survival. TGF-β1 overexpression has been linked to a reduction in peritoneal fluid and peritoneal tissue natural killer (NK) cell and macrophage numbers leading to suppressed scavenger function in the peritoneum (Hull et al. 2012; Mizumoto 1996; Dou et al. 1997). This could limit the clearance of retrograde menstrual tissue within the peritoneal cavity and may lead to a greater chance of ectopic endometrial cell survival.

TGF-β regulation of ectopic endometrial cell attachment onto the peritoneum

Adhesion of human endometrial cells to mouse peritoneum is increased on exposure to TGF-β1 in an *in-vitro* co-culture attachment assay (Beliard et al. 2003), but the mechanism by which TGF-β1 treatment increases ectopic cell adhesion is unclear. It may be induced by altered expression of cell surface adhesion molecules on the peritoneal mesothelial cells, changes in the morphology of the peritoneal mesothelial cells exposing the underlying peritoneal tissue or altered expression of cell surface adhesion molecules on the endometrial cells, or a combination of some or all of the above (Beliard et al. 2003). As discussed previously, the mechanism of ectopic endometrial cell attachment to the peritoneum and the site of ectopic endometrial cell attachment, either directly to the peritoneal mesothelium or to

the underlying connective tissue, is not fully understood (Dunselman et al. 2001). Another study using a functional co-culture adhesion assay showed conflicting results, with exposure to 5ng TGF-β1 significantly increasing attachment of EM42 endometrial epithelial cells to LP9 peritoneal mesothelial cells, but this was not reproducible for the primary endometrial epithelial cells (Liu et al. 2009). Additionally, exposure to 10ng TGF-β1 significantly reduced the attachment of primary endometrial epithelial cells to LP9 peritoneal mesothelial cells, but this was not reproducible for the EM42 cell line (Liu et al. 2009); in this study, the authors pre-treated the endometrial epithelial cells with TGF-β1. An interesting follow-up study would be to repeat the attachment assay with pre-treated peritoneal mesothelial cells, as these cells will also be in direct contact with the peritoneal fluid and hence the increased TGF-β1 concentrations in women with endometriosis. Moreover the peritoneal mesothelium is a key defensive barrier: therefore it is more likely that changes within the peritoneal mesothelial cells than changes to the ectopic endometrial cells increase ectopic cell adhesion and invasion.

TGF-β regulation of ectopic endometrial cell invasion into the peritoneum

Studies have shown that TGF-β1 enhances ectopic endometrial cell invasion into peritoneal tissue during the development of endometriosis lesions (Liu et al. 2009). Using a three-dimensional cell invasion assay model, Liu et al. demonstrated that TGF-β1 dose-dependently increases invasion of EM42 endometrial epithelial cells and primary endometrial epithelial cells though a monolayer of LP9 peritoneal mesothelial cells, and this effect is inhibited by addition of a TGF-βR1 antagonist (Liu et al. 2009). The endometrial epithelial cells were pre-treated with either TGF-β1 and/or TGF-βR1 antagonist, showing the effects of TGF-β1 on ectopic endometrial cells. This study demonstrates that TGF-β1 is able to increase endometrial epithelial cell invasion and results suggest that TGF-β1 maybe inducing epithelial to mesenchymal transition (EMT) within these cells, which would explain the increased migratory and invasion capacity (Liu et al. 2009). EMT within the peritoneal

mesothelial cells has also been discussed in the pathophysiology of endometriosis by increasing ectopic endometrial cell attachment or invasion into the peritoneum through disruption of the mesothelial monolayer (Dunselman et al. 2001; Weusten et al. 2000; Demir et al. 2004).

TGF-β1 is the most well-known inducer of EMT and one group has demonstrated that TGF-β1 may be a cause of EMT within the ectopic endometrial epithelial cells of endometriosis lesions in baboons and this was linked to increased cellular contractility and lesion-associated fibrosis (Zhang et al. 2016a) In a follow-up baboon study, TGF-β1 was confirmed to induce EMT within ectopic endometrial epithelial cells and immunohistochemical analysis has shown that concentrations of TGF-β1 and pSmad3 were correlated with the extent of fibrosis (Zhang at al. 2016b).

TGF-β1 regulation of peritoneal immune cell activity

TGF-β1 autocrine and paracrine signalling within peritoneal macrophage populations were shown to play an essential role in the development of endometriosis lesions (Dou et al. 1997). In-vitro functional assays showed that TGF-β1 regulates macrophage DNA synthesis and cell proliferation, macrophage cell-cell interaction and mRNA expression of several macrophage cell surface adhesion molecules, including: integrins α2, α3, α4, αv, β1, β6 and platelet-endothelial cell adhesion molecule-1 (Dou et al. 1997). Blocking TGF-β expression and signalling in these cells using TGF-β1 antisense oligomers prevented these effects (Dou et al. 1997). TGF-β expression by peritoneal macrophages may also regulate integrin expression both within the ectopic endometrial cells and the peritoneal mesothelial cells, contributing to ectopic endometrial cell attachment to the peritoneum, however this mechanism has not yet been discussed within endometriosis literature. Interestingly, peritoneal endometriosis lesions from Tgfb1-null mice contained significantly reduced numbers of macrophages, when compared to wild-type control mice, suggesting that TGF-β1 is responsible for the recruitment of peritoneal macrophages into endometriosis lesions (Hull et al. 2012). TGF-β

signalling has been shown to promote M2-type macrophages activation, which are involved in inflammation, tissue repair and promote removal of apoptotic cells (Depeng Gong 2012). Recently it was shown that M1-type macrophages suppress endometriotic lesion development, whereas M2-type macrophages, associated with wound healing and tissue remodeling, enhance lesion development (Bacci et al. 2009).

Decreased NK cell activity within the peritoneal cavity in women with endometriosis has been attributed to increasing concentrations of TGF-β within the peritoneal fluid (Mizumoto 1996). Furthermore, peritoneal fluid from women with endometriosis, or treatment with TGF-β, inhibited the development of mice embryos and this was attributed to the decrease in NK cell activity, although again the TGF-β isoforms measured or used in treatments is unknown (Mizumoto 1996). Nevertheless, these results do suggest that increasing TGF-β levels in the peritoneal fluid, and potentially the endometrium of women with endometriosis, may have an adverse effect on fertility.

TGF-β1 regulation of ectopic endometrial cell proliferation

Traditionally, TGF-β1 has been known to have anti-proliferative effects on epithelial cells but proliferative effects on stromal cells (Seoane 2006). In a mouse model of endometriosis, Hull et al. (2012) demonstrated no change in endometrial epithelial or stromal cell proliferation in Tgf-b1 deficient mice, compared to wild-type mice, using BrdU staining. The mouse model utilised within this study used endometrial tissue from human subjects and therefore the endometrial cells themselves were not Tgf-β1 deficient, which may explain why no change in cell proliferation was observed. Supporting the finding that TGF-β1 does not regulate endometriotic cell proliferation, an in-vitro assay demonstrated TGF-β1 exposure has no effect on endometrial epithelial cell proliferation, either in primary endometrial epithelial cells or in the EM42 endometrial epithelial cell line, across several concentrations (Beliard et al. 2003). Conversely, in another study TGF-β1 was shown to increase protease activated

receptor 2 (*PAR2*) mRNA expression and activation in endometrial stromal cells (Saito et al. 2011), and *PAR2* has been shown to induce proliferation of endometrial stromal cells (Yasushi Hirota 2005). Par2-deficient mice also develop smaller and fewer endometriosis lesions than wild-type counterparts, suggesting a role for TGF-β1-mediated Par2 expression in ectopic endometrial cell proliferation (Osuga et al. 2008).

TGF-β1 regulation of neoangiogenesis

Neoangiogenesis is a critical step in the pathophysiology of endometriosis and studies have demonstrated that blocking angiogenesis can block the establishment or growth of endometriosis lesions in a murine model of endometriosis (Laschke 2005; Hull et al. 2003). At a macroscopic level, lesions have been shown to be highly vascularised with new vessels developing from the surrounding peritoneum in hamsters (Overton et al. 2007). Vascular endothelial growth factor-A (VEGF-A) is the most potent angiogenic factor, which is increased in the peritoneal fluid of women with endometriosis compared to women without the disease (McLaren et al. 1996; Kupker et al. 1998; Young et al. 2015). TGF-β1 is an established regulator of VEGF expression in several cell types and overexpression of TGF-β and VEGF has been implicated in neoangiogenesis of several cancers (Kaminska et al. 2005). We have shown that the protein concentrations of VEGF-A in the peritoneal fluid of women with and without endometriosis correlate with concentrations of TGF-β1, suggesting a regulatory role for TGF-β1 in the peritoneal expression of VEGF-A (Young et al. 2015). In the same study, we demonstrated that TGF-β1 may be responsible for the increase in secretion of VEGF-A from the peritoneal mesothelium through the Inhibitor of DNA Binding Protein 1 (*ID1*) pathway, in a similar mechanism to several epithelial cancers, thus contributing to the vascularisation of endometriosis lesions (Young et al. 2015).

TGF-β1 regulation of ectopic endometrial and peritoneal mesothelial cell metabolism

Ectopic endometrial tissue must survive in a hypoxic environment during peritoneal transport, attachment and invasion into the peritoneum, much like metastatic cancer cells. During tumour development and metastasis, glycolysis is initially used for energy production, owing to the hypoxic conditions. Although tumours will eventually develop a blood supply and hence a supply of oxygen, tumour cells continue to use glycolysis as their main source of energy production and this phenotype is often referred to as the 'Warburg effect' (Gatenby & Gillies 2004). Side effects of glycolysis include an increase in cell proliferation and motility, breakdown of ECM and a resistance to apoptosis all of which contribute towards the progression of the disease (Gatenby & Gillies 2004). The Warburg effect is induced by inflammatory cytokines, including TGF-β1, via the induction of hypoxia inducible factor (HIF)-1α protein expression under normoxic conditions (Fosslien 2008; Guido et al. 2012).

There are observations in the literature that suggest ectopic endometrial tissue is using glycolysis as a means of energy production, such as absence of glycogen deposits, the presence of small mitochondria and resistance to apoptosis (Jones et al. 2009). Furthermore, studies have shown *HIF-1α* to be expressed in endometriosis lesions and HIF-1α mRNA and protein expression levels are significantly increased in lesions when compared to matched eutopic endometrium and healthy control endometrium (Ren et al. 2007; Wu et al. 2007), although these studies did not link the reported findings to changes in endometriotic cell metabolism.

We described for the first time potential changes in the cellular metabolism of ectopic endometrial tissue and the surrounding peritoneal tissue of endometriosis lesions, similar to that of the Warburg effect seen in tumourigenesis (Young et al. 2014a). In this study we described significantly higher levels of lactate within the peritoneal fluid of women with endometriosis and we reported a significant positive correlation between concentrations of lactate and TGF-β1. These findings were backed up with work *in vitro* demonstrating that

TGF-β1 increases lactate concentrations in primary peritoneal mesothelial cells and a mesothelial cell line, suggesting that TGF-β1 may regulate changes in cell metabolism that may fuel ectopic endometrial cell survival and endometriosis lesion development (Young et al. 2014a). In a follow up study we demonstrated that TGF-β1 induces changes in the metabolic phenotype through the inhibitor of DNA-binding protein 2 (*ID2*) pathway (Young et al. 2016).

The clinical significance for TGF-β1 in peritoneal endometriosis

Therapeutic moderators of TGF-β expression in endometriosis

GnRH analogues (GnRHa) are commonly used in the medical management of endometriosis (Panay 2008). While the primary effect is in blocking the production of sex steroids from the ovary, endometrial stromal cells express GnRH receptors and GnRHa can act directly on these cells, inducing changes in gene expression, including the expression of TGF-β isoforms and their receptors (Chegini et al. 2003). Therefore, treatment with GnRHa may have additional efficacy in the treatment of endometriosis by decreasing the expression and signalling of TGF-β. TGF-β concentrations in the peritoneal fluid from women with endometriosis was significantly reduced after 4 months of treatment with a GnRHa, although the *TGFB* isoforms measured were not reported (Kupker et al. 1998). It is not clear how much of this effect is directly mediated by GnRH or indirectly through estrogen removal. Functional studies have looked at the effects of GnRHa and TGF-β1 exposure on the expression of fibronectin by endometrial epithelial cells and stromal cells *in vitro*. Microarray results demonstrated that *TGFB1* significantly increased fibronectin expression, while GnRHa significantly decreased fibronectin gene expression (Chegini et al. 2003). The authors concluded that as fibronectin is an essential component in the attachment of ectopic endometrium to the peritoneum, this might be a mechanism by which GnRHa therapies influence the development of endometriosis lesions (Chegini et al. 2003). In a follow-up

study, Luo et al. demonstrated that GnRHa could inhibit phosphorylation of Smad3 in endometrial stromal cells, suggesting that GnRHa therapies may block *TGF-β* signalling in endometrial cells and potentially other cells expressing the GnRH receptors, however whether this is a direct effect of blocking Smad3 phosphorylation by the TGF-βRI or indirect by inhibition of TGF-β ligand and receptor expression is unclear (Luo et al. 2003a).

TGF-β polymorphisms in women with endometriosis

Although the exact aetiology of endometriosis remains unclear, genetic predisposition is thought to play a role. Twin studies have pointed to a genetic component (Montgomery et al. 2008) and women who have a first-degree relative with endometriosis have an increased chance of developing endometriosis themselves (Giudice & Kao 2004). Several studies have investigated polymorphisms in the *TGFB1* in women with endometriosis, to try and explain the genetic component of this complex disease. The *TGFB1*-509C/T polymorphism is the most commonly researched polymorphism of the *TGFB1* gene in the context of endometriosis, as this polymorphism is the main determinant of plasma TGF-β1 concentrations (Grainger et al. 1999). The results of these studies are inconsistent, with several studies demonstrating *TGFB1* polymorphisms to be associated with endometriosis, where other studies found no association (Lee et al. 2011; Kim et al. 2010; Hsieh et al. 2005). A recent meta-analysis of the association of the *TGFB1*-509C/T polymorphism and the occurrence of endometriosis found no significant relationship (Zhang et al. 2012). Other polymorphisms, such as the *TGFB1*-868T/C, which has been associated with early-stage endometriosis in Korean women (Lee et al. 2011), may be of future interest in endometriosis research with regards to its functional impact on endometriosis lesion development and as a candidate gene marker for endometriosis susceptibility.

Several genome-wide association studies have now been performed to further investigate the genetic predisposition associated with endometriosis. A meta-analysis of these studies has identified eight gene loci to be of possible significance in the pathophysiology of

endometriosis (Rahmioglu et al. 2014). However, none of these gene loci belonged to the TGF-β superfamily, indicating that there is unlikely to be a direct genetic linkage resulting from the TGF-β superfamily (Rahmioglu et al. 2014).

SUMMARY

TGF-β regulates a variety of cellular functions including cell proliferation, cell adhesion, cell migration, cell differentiation, apoptosis, angiogenesis and immune cell function. TGF-β is overexpressed in the peritoneal fluid of women with endometriosis compared to women without disease and expression may also be increased in serum, peritoneum, and eutopic endometrium. Although the expression pattern of TGF-β is documented in the endometriosis literature, less is reported regarding the functional role(s) that TGF-β plays in the development and maintenance of endometriosis lesions. However, new mechanistic studies have recently implicated overexpression of TGF-β in several stages of endometriosis lesion development.

Studies have shown that increased levels of TGF-β1 may be responsible for the impaired immune surveillance within the peritoneum of women with endometriosis owing to its ability to decrease NK cell activity. This decrease in immune surveillance may facilitate ectopic endometrial cell survival within the peritoneal cavity. Furthermore, aberrant TGF-β1 expression within eutopic endometrium and peritoneal fluid of women with endometriosis may increase apoptosis resistance in endometrial cells, further fuelling ectopic endometrial cell survival. Attachment of ectopic endometrial cells to the surface of peritoneum and invasion of ectopic cells through the peritoneal mesothelium may increase on exposure to TGF-β1, although the mechanisms governing this and the cell types altered by TGF-β signalling, either peritoneal mesothelial cell or ectopic endometrial cell or both, are not entirely clear. TGF-β1 overexpression may also contribute to ectopic cell survival, invasion

and angiogenesis though changes in cell metabolism to mimic that of cancer cell metabolism, and finally TGF-β1 may also regulate neoangiogenesis though expression of VEGF-A.

Mouse studies using the TGF-β1-null phenotype have given particular insights into the processes that TGF-β1 is likely to regulate during endometriosis lesion formation. Reduced numbers of macrophages and myofibroblasts in endometriosis lesions from Tgfb1null mice suggest TGF-β1 regulation of immune and inflammatory responses. However, there were no observed changes in cell proliferation or blood vessel density, suggesting that TGF-β1 may not be essential for cell growth or angiogenesis within peritoneal endometrioses lesions, contradicting the above observations. However, the overall reduced size and number of endometriosis lesions in Tgfb1-null mice compared to wild type mice does indicate that targeting the TGF-β pathway may be of potential therapeutic interest.

The overexpression of TGF-β1 in the endometriosis microenvironment may contribute to the pathophysiology in a similar fashion to its oncogenic effects during tumourigenesis, by inducing changes in cellular metabolism, increasing cell invasion and initiating neoangiogenesis. Indeed, the same processes that induce TGF-β's tumour promoting activity may also be critical in endometriosis lesion development and a switch in TGF-β signalling, from tumour suppressor to tumour promoter, may help explain why some women develop endometriosis and others do not. Endometriosis is associated with an increased risk of several cancers, including ovarian cancer, breast cancer and non-Hodgkin's lymphoma, therefore it is likely that the same causalities or environmental factors which predispose to the development of endometriosis lesions contribute to the onset of these cancers and vice versa (Kokcu 2011). This review highlights a key role for TGF-β1 in the pathophysiology of peritoneal endometriosis and suggests that therapeutic agents which target TGF-β1 expression or its downstream signalling targets may be beneficial in the prevention and/or treatment of peritoneal endometriosis.

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Authors' roles

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Conflict of Interest

The authors declare they have no conflicts of interest.

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Figure legends

Figure 1. The TGF-β-Smad signalling pathway.

Transforming growth factor β (TGF-β) ligands bind to the receptor TGF-βRII, resulting in a conformational change that results in the recruitment of TGF-βRI. The TGF-β receptor complex phosphorylates intracellular receptor regulated Smad2 and Smad3, which form a dimer before coupling with Smad4 and trans-locating to the nucleus where these transcription factors regulate gene expression. TGF-β ligands may also bind the TGF-βRIII, which can present these ligands to the TGF-βRII. TGF-β signalling through the Smad pathway is known to have impacts on cell growth, angiogenesis, cell differentiation, apoptosis, invasion, immune cell recruitment and metabolism. Figure is adapted from Schmierer and Hill (2007).

Figure 2. Schematic representation of the potential roles of TGF-β in the recognised steps leading to the establishment and progression of peritoneal endometriosis.

HIF-1α = hypoxia inducible factor α, BAX = BCL2-associated X protein, EMT = epithelial to mesenchymal transition, ID1 = inhibitor of DNA binding 1, ID2 = inhibitor of DNA binding 2, VEGF = vascular endothelial growth factor, NK=natural killer.

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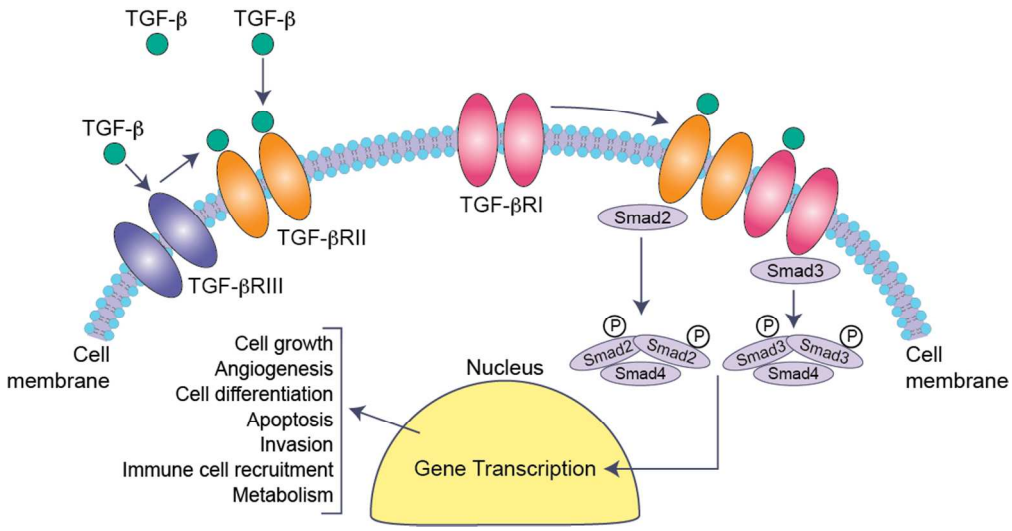

Figure 1 TGF-β-Smad signalling pathway. TGF-β ligands bind to TGF-βRII, resulting in a conformational change that results in the recruitment of TGF-βRI. The TGF-β receptor complex phosphorylates intracellular receptor regulated Smad2 and Smad3, which form a dimer before coupling with Smad4 and trans-locating to the nucleus where these transcription factors regulate gene expression. TGF-β ligands may also bind the TGF-βRIII, which can present these ligands to the TGF-βRII. TGF-β signalling through the Smad pathway is known to have impacts on cell growth, angiogenesis, cell differentiation, apoptosis, invasion, immune cell recruitment and metabolism. Figure is adapted from Schmierer and Hill 2007.

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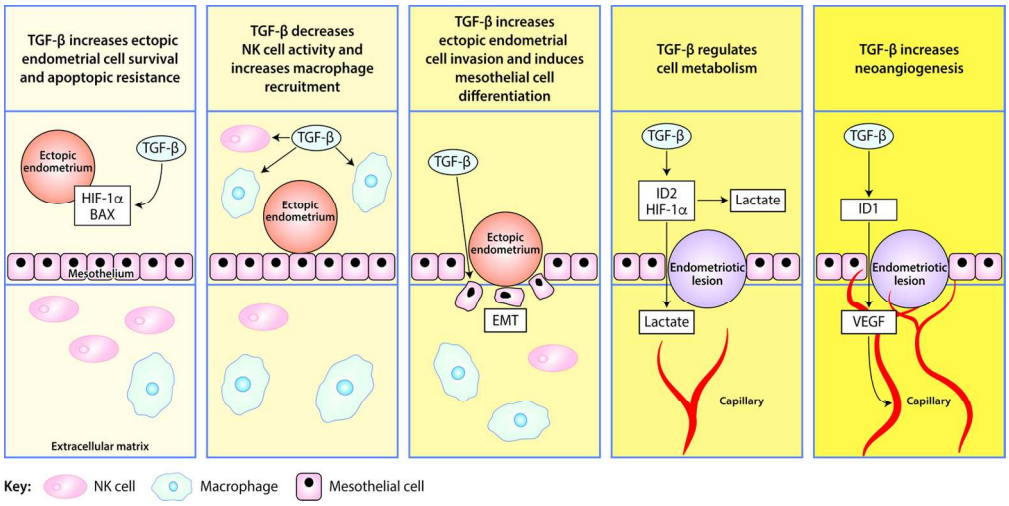

Schematic representation of the potential roles of transforming growth factor β in the recognised steps leading to the establishment and progression of peritoneal endometriosis. TGF-β = transforming growth factor β; HIF-1α = hypoxia inducible factor α, BAX = BCL2-associated X protein, EMT = epithelial to mesenchymal transition, ID1 = inhibitor of DNA binding 1, ID2 = inhibitor of DNA binding 2, VEGF = vascular endothelial growth factor.

142x70mm (300 x 300 DPI)

Ligand	Protein / mRNA	Experimental tissue	Control clinical group	Changes in ligand concentrations in endometriosis	Reference
TGF-β1	Protein	Peritoneal fluid	Pelvic pain	Significantly increased $p<0.05$	Young et al., 2014a
TGF-β	Protein	Peritoneal fluid	No pathology	Significantly increased $p<0.005$	Kupker et al., 1998
TGF-β	Protein	Peritoneal fluid	Infertility	Significantly increased $p<0.001$	Pizzo et al., 2002
TGF-β1	Protein	Peritoneal fluid	No pathology	Significantly increased $p<0.05$	Oosterlynck et al., 1994
TGF-β1	Protein	Peritoneal fluid	Pelvic pain	Significantly increased $p<0.05$	Young et al., 2014b
TGF-β2	Protein	Peritoneal fluid	Pelvic pain	No significant change	Young et al., 2014b
TGF-β3	Protein	Peritoneal fluid	Pelvic pain	No significant change	Young et al., 2014b
TGF-β1	mRNA	Peritoneum	Pelvic pain	Significantly increased $p<0.05$	Young et al., 2014b
TGF-β2	mRNA	Peritoneum	Pelvic pain	No significant change	Young et al., 2014b
TGF-β3	mRNA	Peritoneum	Pelvic pain	No significant change	Young et al., 2014b
TGF-β	Protein	Serum	Infertility	Significantly increased $p<0.001$	Pizzo et al., 2002
TGF-β1	Protein	Serum	Infertility	Significantly increased $p<0.0001$	Lee et al., 2011

Table1. Studies that have measured transforming growth factor (TGF)-β ligand concentrations in women with endometriosis.

Stage of endometriosis lesion development	Function of increased TGF-β1 in endometriosis lesion development	Species	Reference
Immune cell activity	Increased macrophage proliferation	Human	Dou et al., 1997
	Increased macrophage recruitment	Mouse	Hull et al., 2012
	Decreased natural killer cell activity	Human	Mizumoto et al., 1996
Cell survival	Increase in anti-apoptopic factors in eutopic endometrial tissue	Human	Johnson et al., 2005
	Increase in anti-apoptopic factors in ectopic endometrial tissue	Human, mouse	Seoane et al., 2006
	Changes to ectopic endometrial cell metabolism linked to apoptosis resistance	Human	Young et al., 2014a
Cell attachment	Increased attachment of endometrial cells to mouse peritoneal tissue	Human	Beliard et al., 2003
	Increased attachment of endometrial epithelial cells to peritoneal cells	Human	Liu et al., 2009
Cell invasion	Increased invasion of endometrial epithelial cells through peritoneal mesothelial cells	Human	Liu et al., 2009
	Disruption of the peritoneal mesothelial cell monolayer, allowing for ectopic cell invasion	Human	Dunselman et al., 2001; Demir et al., 2004
	Changes to ectopic endometrial cell metabolism linked to increased cell invasion	Human	Young et al., 2014a
Angiogenesis	Increased expression of angiogeneic factors from the peritoneal mesothelium	Human	Young et al., 2015.

Table 2. Functions that an increase in TGF-β1 has been associated with in the development of peritoneal endometriosis lesions.